

**CENTER FOR DRUG EVALUATION AND  
RESEARCH**

*APPLICATION NUMBER:*

**22-080**

**PHARMACOLOGY REVIEW(S)**



**DEPARTMENT OF HEALTH AND HUMAN SERVICES  
PUBLIC HEALTH SERVICE  
FOOD AND DRUG ADMINISTRATION  
CENTER FOR DRUG EVALUATION AND RESEARCH**

**PHARMACOLOGY/TOXICOLOGY REVIEW AND EVALUATION**

**NDA NUMBER: 22-080**  
**SERIAL NUMBER: 000**  
**SUBMISSION DATE : October 16, 2006**  
**PRODUCT: Reclast®**  
**INTENDED CLINICAL POPULATION: Women with postmenopausal osteoporosis**  
**SPONSOR: Novartis Pharmaceuticals Corporation**  
**DOCUMENTS REVIEWED: All nonclinical files (eCTD)**  
**REVIEW DIVISION: Division of Metabolism & Endocrinology Products**  
**PHARM/TOX REVIEWER: Ronald Wange**  
**PHARM/TOX SUPERVISOR: Karen Davis-Bruno**  
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**PROJECT MANAGER: Haley Seymour**

Date of review submission to Division File System (DFS): **July 18, 2007**

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## EXECUTIVE SUMMARY

### I. Recommendations

#### A. Recommendation on Approvability: Approval (AP)

Based on the results of nonclinical pharmacology and toxicology studies, Pharmacology/ Toxicology recommends approval of the NDA for zoledronic acid (Reclast®), 5 mg as 15-minute infusion, for this efficacy supplement for the indication of treatment of postmenopausal osteoporosis (PMO).

The main toxicities identified in nonclinical studies are renal and GI toxicity. The safety margins for these toxicities suggest a low level of safety concern for the proposed 5 mg human IV dose. Clinical monitoring for the toxicities has been performed. The mechanism(s) underlying the serum Ca and P decreases were not clearly established and proper clinical management of these events is required.

B. Recommendation for Nonclinical Studies: No additional nonclinical studies are required.

C. Recommendations on Labeling: Recommended labeling changes have been appended to this Review.

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### II. Summary of Nonclinical Findings

The current application (NDA #22-080) was submitted on October 16, 2006, for the treatment of PMO. Studies submitted to the Division under NDA 21-817 (Reclast® 5 mg, iv, treatment of Paget's disease of bone) were cross referenced.

Zoledronic acid (or zoledronate) is a third generation nitrogen-containing bisphosphonate and a potent inhibitor of osteoclastic activity and bone resorption. The mechanism of action appears to involve inhibition of farnesyl pyrophosphate synthase (FPPS). Inhibition of this enzyme disrupts the function of small molecular weight G proteins, thereby inhibiting multiple cellular processes that are vital for osteoclast function, including: cell migration, cytoskeletal rearrangement, membrane ruffling and vesicle transport. Accumulation of the substrate for FPPS also causes apoptosis by inhibition of mitochondrial adenine dinucleotide translocase.

#### Pharmacology

Zoledronate inhibited *in vitro* osteoclastogenesis, induced rabbit osteoclast apoptosis and altered osteoblast production of OPG and RANKL. The ratio between the IC<sub>50</sub> for calcium incorporation and the IC<sub>50</sub> for calcium release in murine calvaria cultures was 15,000. This suggests that for bone resorption inhibition indications there is very high therapeutic margin with regard to inhibition of bone mineralization.

*In vivo* bone pharmacology studies in ovariectomized (OVX) rats (up to 12-months) and rhesus monkeys (16-month study) demonstrated a dose-dependent increase in bone mineral density and bone strength parameters in OVX animals. Bone turnover and bone remodeling activation frequency were markedly suppressed in trabecular and Haversian bone. There were no clinical

adverse effects in the studies. Bone and bone marrow tissue and cells were normal, and there was no evidence of a mineralization defect, accumulation of osteoid or woven bone.

In the 8-month bone quality study in OVX rats, one single dose of 0.8, 4, 8, 20, 100, 500 ug/kg showed dose-dependent bone protective effects that were transient at the lower doses but persisted for the entire study duration at 100 and 500 ug/kg. There was complete inhibition at 100 and 500 ug/kg of the OVX-induced decreases in proximal tibial BMD and cortical thickness, vertebral compressive strength, and femoral diaphyseal and metaphyseal strength, but there was no significant effect on strength at the femoral neck. The significance of the decreased vertebral and femoral bone strength at the lowest dose of 0.8 ug/kg is unclear. At the 4 ug/kg and 20 ug/kg doses, proximal tibial BMD was increased as compared to OVX but femoral and vertebral strength were not affected. The cause of this apparent discrepancy is unclear.

Safety pharmacology studies indicated that there were no effects on CNS, cardiovascular or respiratory systems.

#### ADME

ADME studies showed a high affinity of zoledronic acid for bone tissue, with rapid binding and slow elimination from this site. The level of bone accumulation was proportional to cumulative dose. Drug in circulation was rapidly eliminated via renal excretion, and there was transient accumulation of drug in the kidney. There was no evidence that the drug is metabolized. Compound was poorly retained in soft tissues such as thymus, kidney, lung, heart, liver, GI tract, compared to bone. Plasma AUC in rats was the same upon i.v. and s.c. dosing. Exposure in TK studies was dose-proportional and there was little accumulation in plasma.

Mannitol, present in the formulation at relatively low levels, is unlikely to affect renal clearance and thus plasma levels of zoledronic acid in humans.

#### General toxicity

In acute i.v. toxicity studies, target organs were kidney, liver and GI tract in rats and dogs. In dogs, a short infusion time of 5 minutes was associated with kidney, GI tract and esophagus lesions while a 15-min infusion did not cause these effects. Serum Ca and P were decreased in dogs with 5- and 15-min infusions. Renal effects in acute infusion studies consisted of tubule basophilia, necrosis, vacuolation, urothelial hyperplasia, and focal inflammation, hemorrhage and congestion. GI findings were inflammation, hemorrhage and congestion in stomach and intestine.

Repeat dose i.v. studies in rats and dogs resulted in effects on kidney, liver, GI tract, and spleen, and irritation at the injection site. The renal toxicity is related to the fact that, like other bisphosphonates, zoledronate is excreted by and retained in the kidney. Most toxicities were at least partially reversible.

Bone changes resulting from the intended pharmacological activity were observed in most repeat-dose studies at the lowest dose and below the NOAEL for other organ toxicity.

In intermittent (2- or 3-weekly) i.v. infusion studies of 2-wk, 13-week, 26-week duration in the dog, renal and GI tract lesions and irritation at the injection site were observed. The renal toxicity was recoverable and included tubular necrosis and degeneration, cortical debris, mineralization, tubular regeneration, inflammation, dilatation, and papillary necrosis. Renal toxicity was exacerbated by repeat dosing with 3-weekly intervals. GI toxicity (congestion, erosion, hemorrhage, inflammation) was observed at higher doses than renal toxicity.

Ca and P were decreased in the single dose and 13-week intermittent infusion studies in dogs at doses below those causing microscopic renal toxicity. The exact mechanism of the disturbance of Ca and P metabolism is unclear but is probably related to the pharmacologic activity of the compound to suppress bone resorption and possibly an effect on kidney tubule function and direct Ca binding.

Safety margins based on NOAEL levels for renal toxicity and (cumulative) AUC or  $\text{mg}/\text{m}^2$  comparison with the 5 mg clinical dose were derived from acute and repeat-dose i.v. (bolus and infusion) dose studies in rats and dogs. The most relevant safety margins for the microscopic renal toxicity based on AUC and single dose animal data were in the range of 1.5x-10x the intended 5 mg clinical dose. The lowest margins (1.5x) were from acute rat studies with bolus injection and large dose separation. In single dose and multiple intermittent dose infusion studies in rats and dogs, safety margins were larger and ranged from 4x-13x. The safety margins are acceptable and support the use of the proposed human 5 mg dose for PMO. The effect on serum Ca and P in dogs was seen at <10 times human exposure (AUC) and these events if they occur need proper clinical management.

#### Genotoxicity, carcinogenicity, reprotoxicity

There was no evidence of mutagenicity in a standard battery of genotoxicity tests: Ames test, an *in vitro* Chinese Hamster cell V79 assay, an *in vitro* Chinese Hamster Ovary clastogenicity assay and an *in vivo* rat micronucleus assay.

Carcinogenicity studies of 2-year duration were carried out using the oral gavage route in mice and rats. In rats, there was no evidence of carcinogenicity. In mice, an increased incidence of Harderian gland adenomas/adenocarcinomas was observed in males at 0.1 and 1.0 mg/kg and in females at doses 0.3 and 1 mg/kg.

Reproductive toxicity studies were performed by the subcutaneous route in rats and rabbits. Dystocia and periparturient mortality were observed in the Segment I rat study probably resulting from drug-related hypocalcemia. The teratogenicity observed in the Segment II rat study may have been due to a decrease in serum calcium levels and/or binding to fetal bone.

#### Conclusion

Taken together, the nonclinical pharmacology and toxicology data submitted to NDA 21-817 support the safety of a single clinical dose of 5 mg zoledronic acid, i.v., for the treatment of PMO.

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**2.6 PHARMACOLOGY/TOXICOLOGY REVIEW**

**2.6.1 INTRODUCTION AND DRUG HISTORY**

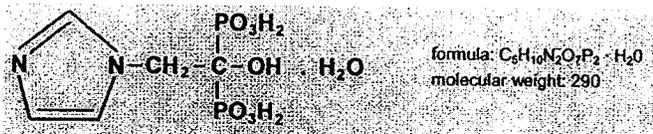
**NDA number:** 22-080  
**Compound:** Zoledronic acid  
**Submission date:** October 16, 2006  
**Sequence number:** 000  
**Review number:** 1  
**Information to Sponsor:** Yes (x) (Labeling comments)  
**Sponsor:** Novartis Pharmaceuticals Corporation, NJ, USA  
**Manufacturer for drug substance:** Novartis Pharma AG, Basel, and Novartis Pharma Stein AG, Stein, Switzerland

**Reviewer name:** Ronald Wange  
**Division name:** Division of Metabolism & Endocrinology Products  
**HFD #:** 510  
**Review completion date:** June 28, 2007

**Drug:**

**Trade name:** Reclast® (zoledronic acid ) Injection  
**Generic name:** Zoledronic acid monohydrate  
**USAN name:** zoledronic acid  
**Code name:** CGP 42446, ZOL446  
**Chemical name:** [1-hydroxy-2-imidazol-1-yl-phosphonoethyl) phosphonic acid monohydrate  
**CAS registry number:** 118072-93-8  
**Molecular formula:** C<sub>5</sub>H<sub>10</sub>N<sub>2</sub>O<sub>7</sub>P<sub>2</sub>·H<sub>2</sub>O  
**Molecular weight:** 290.1 g/mole

Structure:



**Relevant INDs/NDAs/DMFs:** IND 43,240  
 IND 55,831  
 \_\_\_\_\_  
 NDA 21-223 (indication: \_\_\_\_\_ hypercalcemia and \_\_\_\_\_ of malignancy) - Approved  
 NDA 21-386 (indication: treatment of \_\_\_\_\_ bone metastases of solid tumors and \_\_\_\_\_ of multiple myeloma, in conjunction with standard antineoplastic therapy) - Approved

NDA 21-817 (indication: treatment of Paget's disease of bone) - Approved  
DMF \_\_\_\_\_

**Drug class:** Bisphosphonate (bone resorption inhibitor)  
**Indication:** Treatment of postmenopausal osteoporosis  
**Clinical formulation:** Reclast® (zoledronic acid) Injection, solution for intravenous infusion, 5 mg/100 mL. Solution: 5.33 mg zoledronic acid monohydrate (5 mg zoledronic acid), 4950 mg mannitol, 30 mg sodium citrate, \_\_\_\_\_ water for injection  
**Route of administration:** I.V. (15-minute infusion)  
**Dose:** 5 mg \_\_\_\_\_  
**Proposed use:** IV infusion over 15 minutes, one dose per year  
**Pivotal clinical study:** Study 2301: a phase III, 3-year randomized, double-blind, placebo controlled multi-national study of 7736 women aged 65-89 years with either: a femoral neck BMD-T score less than or equal to -1.5 and at least two mild or one moderate existing vertebral fracture(s) or with a BMD T-score of less than or equal to -2.5 with or without evidence of an existing vertebral fracture(s).

**Disclaimer:** Tables and Figures from the Sponsor's electronic NDA submission have been copied for use in this review.

**Studies reviewed within this submission:**

1. RD-2004-01065 - Zoledronic Acid: Inhibitory Activity in a Human Osteoclastogenesis Assay
2. RD-2005-02247 - The Impact of Zoledronic Acid on Bone Marrow-Derived Cells in an Angiogenesis Model
3. DMPK-R0500513 - Comparative Analysis of the *In Vitro* Protein Binding and Its Dependence on the Concentration of Calcium in Rat, Dog and Human Plasma for the Two Bisphosphonates [<sup>14</sup>C]-ZOL446 and [<sup>14</sup>C]-ibandronate
4. PCS(EU)-R0400420 - Analysis of ZOL446 in Plasma and Urine of Male Rats Following a Single Intravenous dose of 0.6 mg/kg [<sup>14</sup>C]-ZOL446

**Studies not reviewed within this submission:** None. All submitted studies reviewed.

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## INTRODUCTION

This NDA is for the treatment of postmenopausal osteoporosis (PMO) with one dose of 5 mg zoledronic acid (15-minute i.v. infusion). An application (NDA 21-817) to market this dosage of zoledronic acid for the treatment of Paget's disease of bone was recently reviewed in DMEP, and was approved on April 16, 2007. Zoledronic acid was previously approved for marketing under the trade name, Zometa® by the FDA (DMEP, ODE II) on August 20, 2001, for the treatment of hypercalcemia of malignancy. On February 22, 2002 the Agency (DODP, ODE I) also approved Zometa® for the treatment of patients with multiple myeloma and patients with documented bone metastases from solid tumors, in conjunction with standard antineoplastic therapy. The approved dose for these cancer-associated treatments was 4 mg, given by  $\geq 15$ -minute i.v. infusion. Sponsor is developing the same compound under the trade name Reclast® for non-oncology indications.

## BACKGROUND

Zoledronic acid (ZOL446) is a nitrogen-containing hydroxy-bisphosphonate. Zoledronic acid has 2 nitrogen atoms in a heterocyclic imidazole ring attached to carbon atom 1, in contrast to other nitrogen-containing bisphosphonates that have a single nitrogen atom in an aliphatic side chain (e.g. pamidronate). Zoledronic acid is a potent inhibitor of osteoclastic bone resorption and skeletal calcium release. Bone resorption can be stimulated by a variety of stimuli (VitD<sub>3</sub>, PTH, PTHrP, PGE<sub>2</sub>). The compound has irritating properties at the site of application, especially when intravenous dosing is employed.

Zoledronate is approved for 3 conditions: (1) bone metastases resulting from primary tumors (AP February 22, 2002), (2) hypercalcemia of malignancy (AP August 20, 2001) and (3) Paget's disease of bone (AP April 16, 2007). Approved doses for cancer-related indications are: 4 mg i.v. infusion every 3-4 weeks for bone metastases (52-72 mg annually), and 4 mg i.v. infusion for hypercalcemia of malignancy. The 4 mg dose is to be given as a single dose infusion over no less than 15 minutes. For hypercalcemia, retreatment may be considered if serum calcium does not return to normal. The approved dose for Paget's disease is 5 mg i.v. infusion over no less than 15 minutes.

The changes in hormone levels associated with the onset of menopause results in an imbalance between osteoclast and osteoblast activity, resulting in net bone resorption. Usual treatment is either with anti-resorptive agents (such as the current drug candidate) or bone anabolic agents.

Known adverse effects of bisphosphonates are GI and renal events. The renal toxicity is believed to be related to the renal excretion of these compounds and has been observed in animals and humans. GI events (esophageal, gastric, intestinal irritation, ulceration, perforation) have been observed in animals and humans. The mechanism of GI toxicity is unclear. In animal studies, both oral and IV dosing can cause GI lesions.

*In vitro* and *in vivo* nonclinical pharmacology, toxicology, and pharmacokinetic (ADME) studies were conducted for NDAs 21-223 and 21-817. For the current NDA, the sponsor submitted additional pharmacology and ADME studies.

## 2.6.2 PHARMACOLOGY

### 2.6.2.1 Brief summary

Zoledronate is a third generation bisphosphonate and a potent inhibitor of osteoclastic bone resorption. Nonclinical data from several *in vitro* and *in vivo* pharmacological models demonstrate that the compound potently inhibits bone resorption.

*In vitro*, zoledronate inhibits bone resorption at concentrations of 0.3-30 nM, and *in vivo* it inhibits bone resorption at doses of 0.3-30 µg/kg. In cultures of murine calvaria the IC<sub>50</sub> value for inhibition of calcium release by zoledronate is approximately 1/100 (0.01x) times the value for pamidronate. In the calvarial cultures, zoledronate and other bisphosphonates also inhibit calcium incorporation. The ratio between the IC<sub>50</sub> for calcium incorporation and the IC<sub>50</sub> for calcium release varies largely, from approximately 3 for etidronate and 500 for pamidronate to 15,000 for zoledronate.

**Table 2-1 Inhibition by zoledronate and reference compounds of calcium release and calcium incorporation in murine calvarial cultures**

compound	Mean IC <sub>50</sub> value (µM) from (n) experiments		ratio (b/a)
	calcium release (a)	calcium incorporation (b)	
zoledronate	0.002 (5)	30 (3)	15000
etidronate	4.0 (3)	10 (2)	3
clodronate	0.4 (3)	50 (2)	125
pamidronate	0.2 (6)	100 (3)	500
alendronate	0.05 (2)	n.d.	n.d.
ibandronate	0.02 (3)	n.d.	n.d.

a: bone resorption stimulated with 20 nM 1,25(OH)<sub>2</sub>D<sub>3</sub> for 72 hr; b: bone mineralization stimulated with 2 mM calcium 1,2-glycerophosphate for 48 hr; n.d. = not determined.

The complete mechanism by which zoledronate reduces bone resorption is not entirely known; however, one mechanism appears to involve disruption of later steps in mevalonate metabolism. Third generation, nitrogen-containing bisphosphonates (such as zoledronate) bind to and inhibit the activity of the enzyme farnesyl pyrophosphate synthase (FPPS). FPPS catalyzes the successive condensation of isopentenyl with dimethylallyl pyrophosphate and geranyl pyrophosphate, key steps in the synthesis of mevalonate metabolites such as farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP). FPP and GGPP are required for the posttranslational modification (prenylation) of small molecular weight G proteins (SMGs) such as Ras, Rab, Rap, Ral, Rho, Cdc42 and Rac. SMGs are important signaling proteins, regulating myriad cell processes important in osteoclast function: cell morphology, cell migration, cytoskeletal rearrangement, membrane ruffling, trafficking of vesicles and apoptosis. Prenylation is required for the correct function of SMGs, because the lipid prenyl group mediates targeting of the SMGs to the appropriate cellular membranes, and also mediates certain protein-protein interactions. It is hypothesized then that the disruption of SMG signaling leads to inhibition of osteoclast function and/or viability. Another potential mechanism of action also stems from the ability of nitrogen-containing bisphosphonates to inhibit FPPS. Inhibition of FPPS causes accumulation of the substrate isopentenyl pyrophosphate (IPP), which can be converted to Apppl (triphosphoric acid 1-adenosin-5'-yl ester 3-(3-methylbut-3-enyl) ester) via aminoacyl-tRNA-synthetases. Apppl induces apoptosis by inhibiting the mitochondrial adenine nucleotide translocase (ANT). Selectivity of bisphosphonates for osteoclasts is a function of the selective

deposition of bisphosphonates to bone, and high local osteoclast exposures to drug when it is released into the region of contact between the osteoclast and the bone during resorption of the bone by the osteoclast.

Empirically, Zoledronate inhibited *in vitro* osteoclastogenesis (RD-2001-00476 & RD-2004-01065), induced rabbit osteoclast apoptosis (RD-1999-0390), and inhibited bone resorption and formation of type I collagen C-telopeptides *in vitro* (RD-2004-01065). Zoledronate also alters osteoblast production of OPG and RANKL, cytokines controlling osteoclast formation and activity (Pan et al, 2004, JBMR 19:147-154).

In the thyroparathyroidectomized rat zoledronate dose-dependently inhibits 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced acute hypercalcemia with an ED<sub>50</sub> of ca. 0.07 µg/kg s.c., and is several orders of magnitude more potent than clodronate or etidronate. The inhibition of hypercalcemia in this model is presumably brought about by inhibition of osteoclastic bone resorption (Study 95/89 IBA).

In short term studies in ovariectomized rats, zoledronate can completely prevent the OVX-induced bone loss at a dose of 0.3 µg/kg given 5x/week for 3 weeks (Studies BS76/1996; 69/94; 86/93 IBA). Long term studies were carried out in ovariectomized rats (12 months) and rhesus monkeys (69 week, i.e., 16 months), of 0.3, 1.5, 7.5 µg/kg/week s.c. (rat) and 0.5, 2.5, 12.5 µg/kg/week, s.c. (monkey). A single dose IV bone quality study was also carried out in the OVX rat (RD 2002-04006). These bone quality studies were reviewed in detail in the P/T review of NDA 21-817 (Gemma Kuijpers, dated: September 21, 2004), and are included herein by cross-reference. In summary, these studies demonstrated a dose-dependent increase in bone mineral density and bone strength parameters in ovariectomized (OVX) rats (up to 12-months) and OVX rhesus monkeys (16-month study). Bone turnover and bone remodeling activation frequency were markedly suppressed in trabecular and Haversian bone. There were no clinical adverse effects in the studies. Bone and bone marrow tissue and cells were normal, and there was no evidence of a mineralization defect, accumulation of osteoid or woven bone.

In the 8-month bone quality study in OVX rats, one single dose of 0.8, 4, 8, 20, 100, 500 ug/kg showed dose-dependent bone protective effects that were transient at the lower doses but persisted for the entire study duration at 100 and 500 ug/kg. There was complete inhibition at 100 and 500 ug/kg of the OVX-induced decreases in proximal tibial BMD and cortical thickness, vertebral compressive strength, and femoral diaphyseal and metaphyseal strength, but there was no significant effect on strength at the femoral neck. The significance of the decreased vertebral and femoral bone strength at the lowest dose of 0.8 ug/kg is unclear. At the 4 ug/kg and 20 ug/kg doses, proximal tibial BMD was increased as compared to OVX but femoral and vertebral strength were not affected. The cause of this apparent discrepancy is unclear.

**Table 3-2 Comparison of the doses used in the long-term monkey and rat studies versus the human 5 mg iv dose / 60 kg body weight**

Experiment	Dose and dose ratios vs human 5 mg dose		
	0.5	2.5	12.5
Monkey: dose µg/kg/week sc (a)	0.5	2.5	12.5
total dose in 69 week study µg/kg	34.5	172.5	862.5
equivalent dose mg/60 kg	2.1	10.4	51.8
ratio: monkey/human 5 mg dose (a)	0.41	2.1	10.4
Rat: dose µg/kg/week sc (a)	0.3	1.5	7.5
total dose in 52 week study µg/kg	15.6	78	390
equivalent yearly mg dose/60 kg	0.94	4.7	23
ratio: rat/human 5 mg dose (a)	0.19	0.94	4.6
Rat: single dose µg/kg iv (b)	0.8	4.0	20
equivalent mg dose/60 kg	0.048	0.24	1.2
ratio: rat/human 5 mg dose (a)	0.007	0.032	0.16
molecular weight of test compound: (a) = 272.1; anhydrous free acid; (b) = 401.6; hydrated disodium salt			

**2.6.2.2 Primary pharmacodynamics**

**Mechanism of action:** Inhibition of farnesyl pyrophosphate synthase with subsequent disruption of signaling via small molecular weight G proteins and inhibition of the mitochondrial adenine nucleotide translocase.

**Drug activity related to proposed indication:** Inhibition of bone resorption via inhibition of osteoclastogenesis and osteoclast activity.

NEW PRIMARY PHARMACOLOGY STUDIES NOT REVIEWED AS PART OF NDAS 21-223, 21-386, OR 21-817

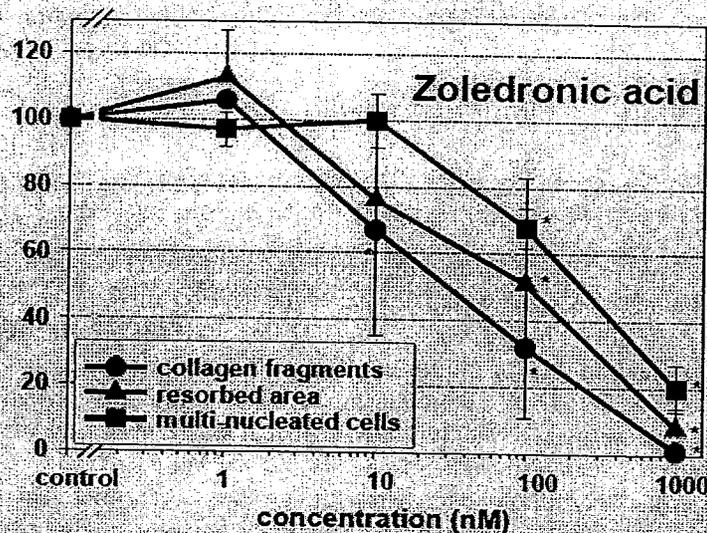
**Title:** RD-2004-01065 Zoledronic acid: Inhibitory activity in a human osteoclastogenesis assay

**Study number:** RD-2004-01065

**Study design:** Human osteoclasts were generated from human peripheral blood mononuclear cells by co-culturing with bovine cortical bone slices in the presence of dexamethasone and a cocktail of cytokines (MCSF, RANKL and TGF- $\beta$ 1). The effect of the bisphosphonate zoledronic acid on osteoclastogenesis and the bone resorbing activity of the osteoclasts was tested by culturing the cells with zoledronic acid in a concentration range between 1 and 1,000 nM. Measures of anti-osteoclastogenesis included: 1) measurement of resorbed area in a pit assay using bovine cortical bone slices and human osteoclasts, 2) measurement of collagen fragments (detected in CrossLaps ELISA) released from resorbed bone into the cell culture media, and 3) the number of TRAP (tartrate-resistant acid phosphatase)-positive multinucleated cells (mature osteoclasts).

**Results:**

**Figure 3-1. Dose response curve for compound zoledronic acid in the human osteoclastogenesis assay**



In the human osteoclastogenesis assay the number of multinucleated cells, bone resorbed area, and collagen fragment release were measured as described in Methods. Zoledronic acid was added at the indicated concentrations from the beginning of cell culture. The results are expressed as % of control and the graph represents a summary of 3 independent experiments, each performed with n=4 per data point. Collagen fragments: ELISA assay for measurement of collagen fragments released from the pits; resorbed area: bone slice area resorbed by cells. \*p<0.05, calculated by the Sigma Plot 8.0 software, unpaired Student t-test



The Summary also notes that multiple *in vitro* and *in vivo* models of angiogenesis have shown that zoledronate (3-100 µg/kg) has anti-angiogenic activity. Study number RD-2005-02247 (reviewed below), which shows that exposure of the VEGF-containing s.c. implant to circulating drug is not required for zoledronate-mediated inhibition of soft tissue angiogenesis, is consistent with Sponsor's proposal that the anti-angiogenic effect of zoledronate is mediated by local effects on bone marrow cells or bone marrow-derived soluble mediators. It is not clear that this activity has any relevance in the treatment of PMO.

**Title: RD-2005-02247: The Impact of Zoledronic Acid on Bone Marrow-Derived Cells in an Angiogenesis Model**

**Study number:** RD-2005-02247

**Study design:** The Sponsor had previously found that zoledronic acid has anti-angiogenic activity in a growth factor implant model. Given that zoledronic acid is rapidly sequestered to bone, they hypothesize that bone marrow cells (e.g. endothelial precursors, inflammatory cells) are the primary target of the anti-angiogenic activity of zoledronic acid. The goal of this study was to identify the bone marrow cell type(s) that mediate the anti-angiogenic effect of zoledronic acid.

**Study A – Ability of bone sequestered zoledronic acid to inhibit angiogenesis:** One group of mice received a single dose of drug (100 µg/kg s.c.) 3 days prior to implantation of angiogenesis chamber containing agar with or without 2 µg VEGF. Another group received daily s.c. injections of drug (100 µg/kg) for 4 days beginning on the day of chamber implantation. Four days after implantation of the chamber, the animals were sacrificed and the chambers removed. Tissue in the chamber was removed, weighed, homogenized in RIPA buffer and both hemoglobin and Tie-2 protein (expressed at high levels in the vascular epithelium) content measured.

**Study B – Effect of zoledronic acid on inflammatory & endothelial cell numbers in blood or bone marrow:** Mice were injected once s.c. with zoledronic acid (100 µg/kg) or EDTA (control). After 7 days the mice were sacrificed, blood and bone marrow were isolated. The levels of lymphocytes, monocytes and granulocytes in blood were counted. Bone marrow samples were analyzed by FACS analysis for macrophages (α-F480 Ab staining), B cells (α-CD19 Ab staining), T-cells (α-CD3 Ab staining) and endothelial cells (α-VEGFR2 Ab staining).

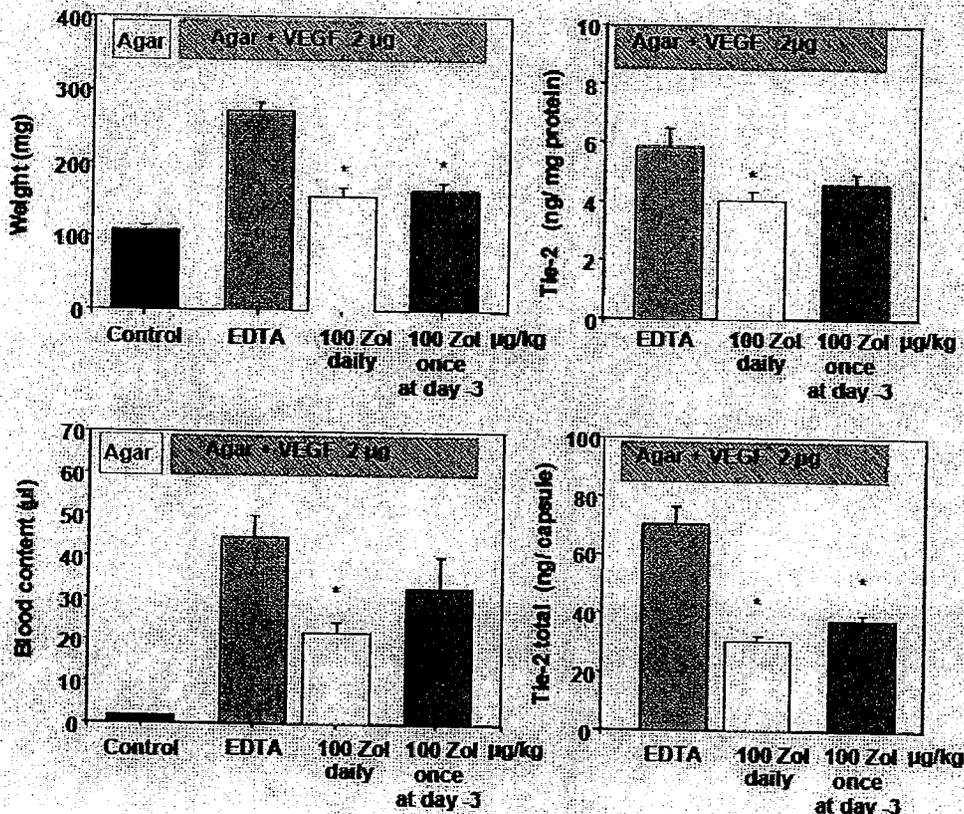
**Study C – Effect of zoledronic acid on inflammatory & endothelial cell numbers in blood, bone marrow and implanted VEGF chambers:** Mice were treated with a single s.c. injection of drug (100 µg/kg zoledronic acid) 3 days prior to implantation of VEGF chambers. Four days after chamber implantation animals were sacrificed, chambers removed and blood, marrow and chamber were assessed for numbers of lymphocytes, monocytes and granulocytes (blood) and macrophages, B cells and vascular endothelial cells as in study B.

**Study D – Identification of bone marrow-derived cells by transplantation of bone marrow cells expressing GFP:** Bone marrow was ablated in recipient C57BL/6 mice by i.p. injection of 30 mg/kg busulfan. Bone marrow from donor mice (C57BL/6 background) that express GFP (green fluorescent protein) under either the actin gene promoter (ubiquitous expression) or Tie-2 promoter (expressed primarily in vascular endothelial cells) were transferred to the recipient mice 24 hr after busulfan treatment. BM-transplanted mice were injected once s.c. with zoledronic acid (100 µg/kg) or EDTA (control) 3 days prior to implantation of VEGF chambers. Four days after chamber implantation animals were sacrificed, chambers removed and marrow and chamber were assessed for numbers of macrophages, B cells, T cells and vascular endothelial cells as in study B.

Results:

Study A

Figure 0-1 Agar chamber model in mice treated with different regimens of zoledronic acid



Study B

A) Blood

	Lymphocytes	Monocytes	Granulocytes
EDTA	57.4% ± 8.9	10.3% ± 1.1	32.3% ± 9.9
Zol	61.6% ± 7.3	9.5% ± 0.7	28.9% ± 7.3

	F480 (Macrophages)	CD19 (B-cells)	CD3 (T-cells)	VEGFR2 (endoth. cells)
EDTA	23.86% ± 1.34	11.45% ± 1.59	5.92% ± 0.7	0.68% ± 0.12
Zol	21.93% ± 1.6	11.38% ± 2.69	5.32% ± 0.93	0.62% ± 0.11

Study C

A) Analysis of blood (n=6)

	Lymphocytes	Monocytes	Granulocytes
EDTA	52% ± 5.9	9.9% ± 0.6	38.1% ± 6.4
Zol	48.5% ± 10.1	7.7% ± 1.3	43.79% ± 11.7

B) FACS analysis of bone marrow (n=4)

	F480 (Macrophages)	CD19 (B-cells)	Tie2 (endoth.cells)
EDTA	23.62% ± 3.3	7.1% ± 0.77	0.48% ± 0.19
Zol	23.14% ± 2.93	7.93% ± 1.41	0.39% ± 0.41

C) FACS analysis of chamber tissue (n=4)

	F480 (Macrophages)	CD19 (B-cells)	Tie2 (endoth.cells)
EDTA	38.75% ± 2.13	5.99% ± 2.13	4.44% ± 1.97
Zol	37.75% ± 2.22	7.86% ± 2.24	2.85% ± 0.73

Study D

Too few GFP-positive cells were recovered from Tie-2-GFP BM donors, so the Sponsor focused on the results from the actin-GFP BM donors.

A) Summary of FACS analysis of bone marrow of actin-GFP –BM transplanted mice implanted with VEGF chambers

		F480 (Macrophages)	CD19 (B-cells)	CD3 (T-cells)	Tie2 (endoth.cells)	GFP (bm derived)
EDTA	Antigen positive	27.86% ± 1.86	9.63% ± 3	7.1% ± 1.46	0.98% ± 0.3	31.98% ± 10.37
	GFP/Antigen double pos	10.36% ± 4.1	2.64% ± 2.2	0.067% ± 0.038	0.029% ± 0.017	
Zol	Antigen positive	28.45% ± 4.42	7.41% ± 2.67	5.22% ± 0.34	0.94% ± 0.13	29.49% ± 8.93
	GFP/Antigen double pos	0.01% ± 2.76	1.96% ± 1.34	0.045% ± 0.154	0.024% ± 0.01	

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**B) FACS analysis of chamber tissue of actin-GFP –BM transplanted mice implanted with VEGF chambers**

		F480 (Macrophages)	CD19 (B-cells)	CD3 (T-cells)	Tre2 (endothl. cells)	GFP (bm derived)
EDTA	Antigen positive	44.51% ± 5.52	4.08% ± 1.44	8.38% ± 1.92	6.67% ± 2.02	43.42% ± 14.97
	GFP/Antigen double pos	16.85% ± 8.3	0.12% ± 0.29	0.44% ± 0.31	0.554% ± 2.99	
Zol	Antigen positive	36.47% ± 3.8	4% ± 0.42	9.24% ± 2.18	6.18% ± 2.16	39.56% ± 16.8
	GFP/Antigen double pos	13.22% ± 4.89	0.17% ± 0.29	0.58% ± 0.49	0.476% ± 0.120	

**Conclusion:** A single dose of 100 µg/kg zoledronic acid administered 3 days prior to VEGF chamber implantation reduced angiogenesis. Experiments to determine whether zoledronic acid mediates its effects via bone marrow-derived cells were inconclusive.

#### 2.6.2.4 Safety pharmacology

It is well known that renal toxicity can occur upon treatment with relatively high doses of bisphosphonates. Renal toxicity is discussed in the toxicology section of this review.

Zoledronate had no significant CNS effects at doses up to 10 mg/kg i.v. in mice. It also has no significant effects on gastrointestinal transit time, drug-induced convulsions, or cardiac and smooth muscle contraction. There were no effects on *in vivo* respiration, hemodynamic and ECG parameters in anesthetized cats.

There were no ECG changes in a 4-week iv toxicity in dog up to 0.2 mg/kg/day, and in 3-month and 6-month iv toxicity studies in dog at doses up to 0.1 or 0.2 mg/kg/day (daily dose equivalent to human dose of 2.5-5 mg, based on mg/m<sup>2</sup>).

The acute phase response and increase of circulating cytokine levels that occurs in some patients treated with iv doses of bisphosphonates is probably related to stimulation of a γδ-T cell subset triggered by accumulation of intermediates in the mevalonate pathway following FPP synthase inhibition (Thompson and Rogers, 2004).

In a fracture healing study, zoledronate (100 µg/kg, i.v.) increased the amount and strength of regenerate bone in tibial osteotomy.

#### 2.6.2.5 Pharmacodynamic drug interactions

No studies performed by Sponsor. There is some indication in the literature that administration of zoledronate to OVX rats following 5 weeks of daily treatment with PTH helps to preserve the PTH-related gains in bone density and bone strength upon discontinuation of PTH. Literature data in the rat also suggests that zoledronate + osteogenic protein (OP-1) enhanced the healing response in a critical size defect model over that achieved with OP-1 alone.

2.6.3 PHARMACOLOGY TABULATED SUMMARY

(Since submission of Paget's NDA [21-817])

2.6.3.1 Pharmacology Overview

Type of study	Test system or protocol	Dose and route of administration	Report number/ Location
<b>Primary Pharmacodynamics</b>			
<i>In vitro</i>			
Effect of zoledronate on the generation and bone resorptive activity of osteoclasts formed from cultured human peripheral blood monocytes.	Formation of active osteoclasts induced by incubating human peripheral blood monocytes with M-CSF, RANKL, TGF-β1 and dexamethasone on bovine cortical bone slices. Osteoclast number, bone resorption area, and release of collagen fragments measured at day 17.	1 - 1000 nM added to culture medium	RD-2004-01065
Effect of zoledronate on the viability, proliferation and osteogenic differentiation of primary human osteoblast-like cells.	Cells from bone biopsies or marrow aspirates cultured on plastic, cell viability, proliferation and stage in the cell cycle measured for up to 35 days. Differentiation assayed by RT-PCR (osteocalcin, BMP-2) and calcified mineral content.	0.05 - 25 μM added to culture medium	_____ (2004)
Effect of zoledronate on the viability, proliferation and osteogenic differentiation of human bone marrow cells.	Human bone marrow cells from surgical specimens cultured on plastic, cell proliferation measured up to 14 days. Differentiation assayed by alkaline phosphatase activity and RT-PCR (BMP-2, BSP-4, cbfa-1, collagen type 1) and calcified mineral content.	10 nM added to culture medium	_____ (2005)
Comparison of the effect of zoledronate on the viability of murine MC3T3-E1 osteoblasts cultured on plastic versus a calcium phosphate disk.	Osteoblasts cultured on calcium phosphate disks coated with zoledronate or on plastic with drug present in the medium for 2 hr, 1 day or continuously. Cell viability and alkaline phosphatase activity measured at day 3.	1 - 50 μM coated onto disks or added to culture medium	_____ (2005)
Indirect measurement of the binding affinity of several bisphosphonates for pure hydroxyapatite crystals <i>in vitro</i> .	Affinity constants calculated from kinetic studies on the inhibition of hydroxyapatite crystal growth using a constant composition potentiostatic method at pH 7.4, 37°C and physiological ionic strength (0.15 M).	0.17 - 0.65 μM <i>in vitro</i>	_____ (2006)
<b>Primary Pharmacodynamics</b>			
<i>In vivo</i>			
Direct measurement of the binding affinity of several bisphosphonates for human bone particles <i>in vitro</i> .	Affinity constants calculated from competitive binding assays with [ <sup>14</sup> C]-labelled alendronate and human bone particles (150 - 180 μm) in 0.2 M Tris-formate buffer at pH 7.2 at 22°C.	64 nM - 5 mM <i>in vitro</i>	_____ (2005)
Endocytic uptake of bisphosphonates by rabbit osteoclasts and murine J774 macrophages <i>in vitro</i> .	Uptake of fluorescent-labelled alendronate and [ <sup>14</sup> C]-labelled zoledronate by osteoclasts and macrophages measured by confocal microscopy and competitive inhibition assays.	25 - 100 μM <i>in vitro</i>	_____ (2005)
Structural analysis of the interaction between bisphosphonate and the active site of recombinant human farnesyl pyrophosphate synthase <i>in vitro</i> .	X-ray crystallographic and calorimetric studies on bisphosphonate binding in the presence and absence of the substrate isopentenyl pyrophosphate.	300 - 500 μM <i>in vitro</i>	_____ (2006)
Formation of a novel cytotoxic ATP analogue (Apppl) induced by bisphosphonates <i>in vitro</i> .	Rat osteoclasts, murine J774 macrophages and rat C6 glioma cells treated with bisphosphonates. Apppl formation assessed by mass spectrometry and NMR; effects on mitochondrial function and induction of osteoclast apoptosis studied.	0.1 - 30 μM <i>in vitro</i>	_____ (2006)
<b>Primary Pharmacodynamics</b>			
<i>In vivo</i>			
Prevention of bone loss associated with inflammatory arthritis in transgenic mice over-expressing human TNFα.	Bone changes assessed by micro-CT, pQCT, histomorphometry, serum deoxyribonitine and serum osteocalcin. Arthritis assessed by clinical signs and joint histology.	100 μg/kg i.p., either once at week 4 or 5x/week during weeks 4-10	_____ (2004)
Maintenance of new bone induced in OVX rats with PTH (1-84) by sequential treatment with zoledronate.	Osteopenic rats first treated with PTH (1-84) at week 25 post-OVX for 5 weeks followed by 5 weeks of zoledronate. Bone changes in the femur and vertebrae measured by DXA, micro-CT and mechanical testing.	12.5 μg/kg/week s.c. for 5 weeks	_____ (2004)

Type of study	Test system or protocol	Dose and route of administration	Report number/ Location
Effect of a single infusion of zoledronate on biochemical markers of bone metabolism in healthy dogs	Changes in bone metabolism parameters measured in serum and urine at weeks 1, 2, 3 and 4 post-dosing.	250 µg/kg i.v. single infusion	(2005)
<b>Secondary Pharmacodynamics</b>			
no additional studies performed			
<b>Secondary Pharmacodynamics</b>			
<i>In vitro</i>			
Soft tissue anti-angiogenic effect of a prior single dose of zoledronate in the growth factor chamber implant model.	Porous chambers containing agar and VEGF implanted s.c. in mice, antigenic response induced in surrounding tissue measured after 5 days by tissue weight, haemoglobin content and Tie-2 level.	100 µg/kg s.c. once 3 days before chamber implantation	RD-2005-02247
<b>Safety Pharmacology</b>			
Effect of zoledronate on fracture healing in a rabbit model of distraction osteogenesis.	Tibial osteotomy followed by 2 weeks distraction performed in young rabbits. Changes in the callus and endochondral bone remodelling tracked for 44 weeks by radiography, DXA, histology and histomorphometry.	100 µg/kg i.v. at surgery and again 2 weeks later	(2004)
Interaction between anabolic (OP-1) and anti-catabolic (zoledronate) agents in the healing of a rat femoral critical defect.	Rats with a 6-mm femoral defect treated with local OP-1 and systemic zoledronate, either as single agents or combined. Bone changes analysed by radiography, pQCT, histology, histomorphometry and mechanical testing.	100 µg/kg s.c. once either at surgery or 2 weeks later	(2005a)
Effect of zoledronate in a rat model of traumatic avascular osteonecrosis of the femoral head.	Avascular osteonecrosis of the femoral head induced by surgical trauma in rats. After sacrifice at 6 weeks, undecalcified specimens were analyzed by radiography, DXA, histology and histomorphometry.	100 µg/kg s.c. either 1 & 4 weeks post-surgery, or additionally 2 weeks before surgery	(2003)
Type of study	Test system or protocol	Dose and route of administration	Report number/ Location
Effect of zoledronate on femoral head architecture in a spontaneous rat model of avascular osteonecrosis.	Approximately 50% of spontaneously hypertensive rats develop osteonecrosis of the femoral epiphysis at 6-9 weeks of age. Four-week-old rats were treated with zoledronate for 15 weeks, femora were collected and analyzed by radiography, DXA, histology and histomorphometry.	50 µg/kg s.c. every 4 weeks or 15 µg/kg every week to give a total dose of 150 µg/kg	(2005b)
Effect of zoledronate on bone ingrowth into porous tantalum implants in dogs.	Porous tantalum implants inserted into the tibiae of dogs and removed 6 weeks later. Bone ingrowth assessed on undecalcified histological sections by back-scattered scanning electron microscopy.	100 µg/kg i.v. single dose post-surgery	(2005)
Effect of zoledronate on peri-implant bone in a dog model of aseptic loosening of an uncemented total hip arthroplasty.	Hip arthroplasty performed in dogs, prosthesis loosening induced by polyethylene particles (5 µm) packed into the femoral component. Bone and soft tissue changes analyzed by radiography, histology, histomorphometry, back-scattered scanning electron microscopy, density traction, x-ray diffraction and mechanical testing.	2 or 10 µg/kg/week s.c. for 26 weeks	(2005)
<b>Pharmacodynamic drug interactions</b>			
no studies performed			

## 2.6.4 PHARMACOKINETICS/TOXICOKINETICS

### 2.6.4.1 Brief summary

#### Human PK/AUC:

AUC in humans at the 5 mg dose = 650 ngxh/mL (Biopharmaceutics Review, NDA #21-817, Sandra Suarez-Sharp). PK evaluation was based on experimental human PK data with 2, 4, 8, 16 mg from bone cancer patients. The AUC value at 5 mg was calculated with a simulation program (WinNonlin) using initial estimates based on data from Study 1101.

#### Protein binding:

##### Binding:

Human: 62%-55%  
 Dog: 70%-44%  
 Rat 94%-85%

**Clearance:**

The inclusion of mannitol in the dosing solution (4950 mg in 100 mL) could affect renal clearance through an diuresis and an effect on Cls and/or CLRa ( $CL_R = fp.GFR + CLs - CLra$ ). However, animal data have shown that both Cls and Cra for zoledronic acid are likely to be very small (\_\_\_\_\_ 2004). Thus, mannitol is unlikely to affect renal clearance and thus plasma levels of zoledronic acid in humans.

**Summary**

- high affinity and slow elimination from bone tissue
- rapid elimination from circulation and soft tissues via renal excretion
- no evidence of biotransformation
- accumulation in bone proportional to cumulative dose.
- exposure dose-proportional in toxicokinetic studies (rat, dog)
- no or little accumulation in plasma
- no effect of gender on the PK/TK

**2.6.4.2 Methods of Analysis**

None submitted to NDA.

**2.6.4.3 Absorption**

No studies submitted.

**2.6.4.4 Distribution**

**FILE: DMPK-R05005-13** Comparative analysis of the *in vitro* plasma protein binding and its dependence on the concentration of calcium in rat, dog and human plasma for the bisphosphonates [<sup>14</sup>C]ZOL446 and [<sup>14</sup>C]ibandronate.

**Study number:** DMPK-R0500513

**Study design:** Defrosted frozen plasma samples from rat, dog and human were spiked with 2, 20, 200 or 2000 ng/mL of test item (either <sup>14</sup>C-labeled zoledronic acid (ZOL446) or <sup>14</sup>C-labeled ibandronate) in the presence or absence of CaCl<sub>2</sub> (0, 2 and 4 mM) or EDTA (0, 2 and 4 mM). Samples were incubated for 30 min at 37°C. The unbound fraction was collected by ultrafiltration and <sup>14</sup>C measured by liquid scintillation counting.

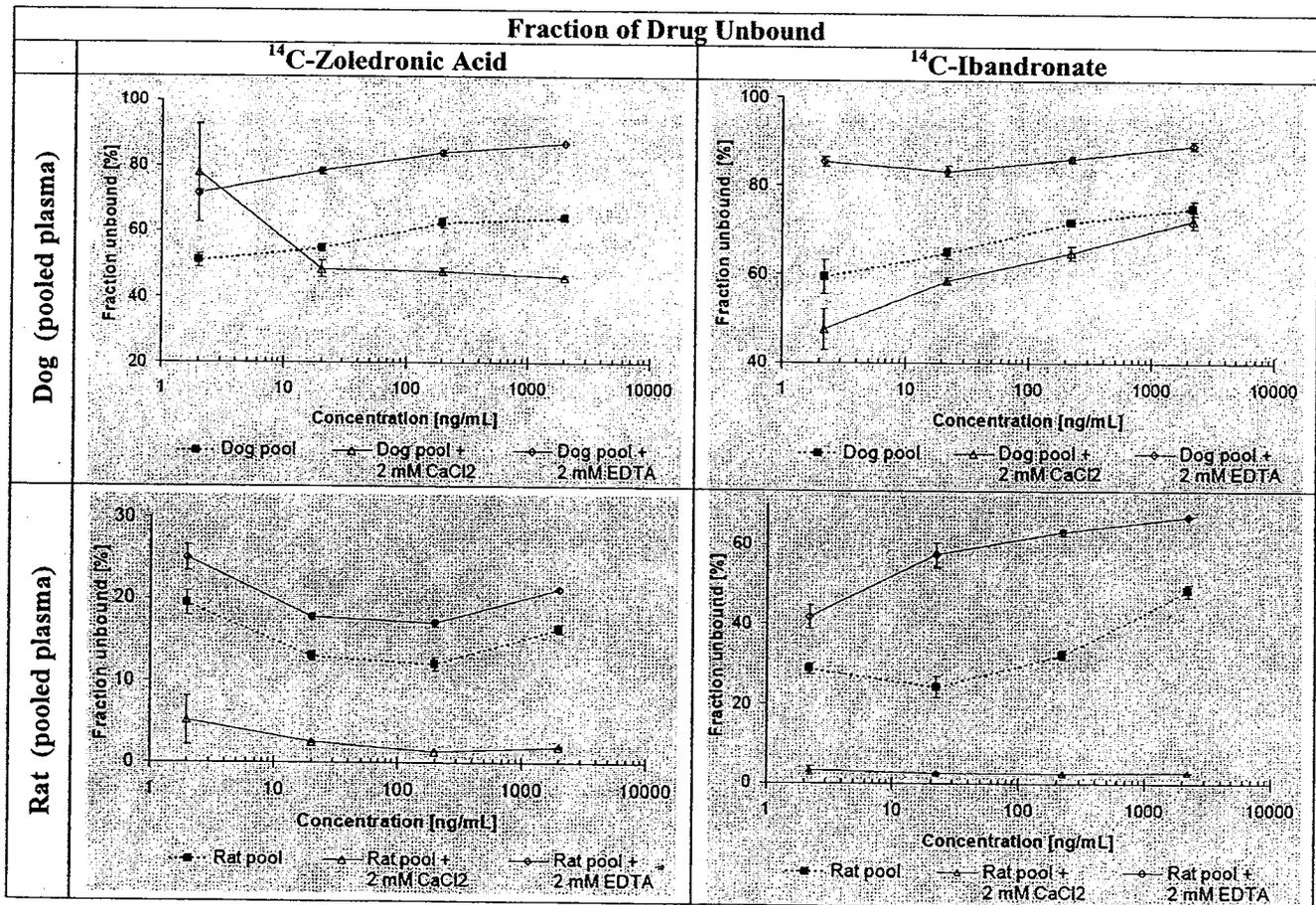
**Results:**

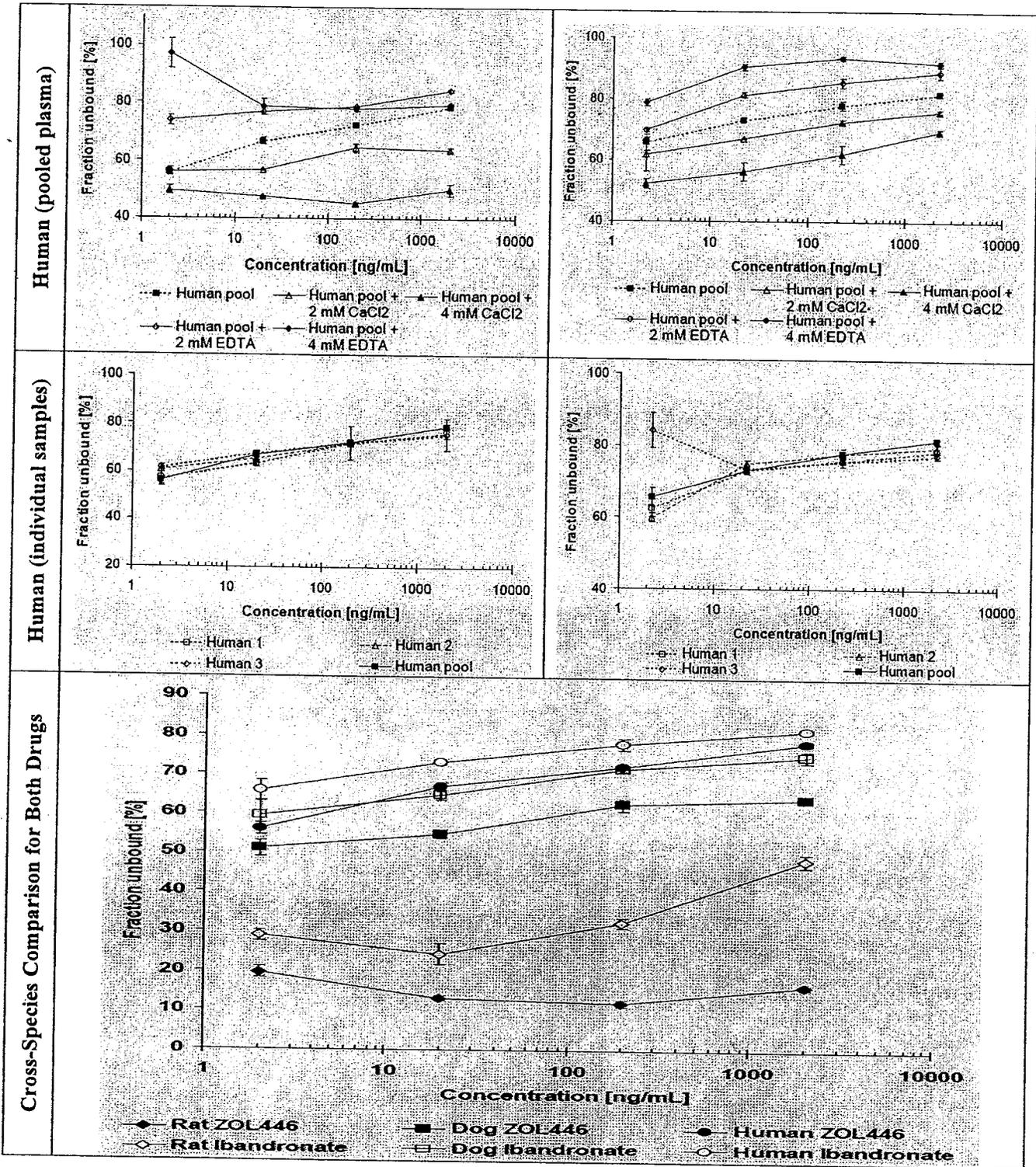
**Table 6-7 Mean unbound fractions and SDs of [<sup>14</sup>C]ZOL446 for dog, rat and human**

Matrix	Nominal plasma concentration			
	2000 ng/ml	200 ng/ml	20 ng/ml	2 ng/ml
Dog pool	64.0 ± 0.7	62.5 ± 1.7	54.7 ± 1.1	50.9 ± 2.1
Dog pool + 2 mM CaCl <sub>2</sub>	46.1 ± 0.4	47.6 ± 1.2	48.4 ± 2.4	77.7 ± 15.1
Dog pool + 2 mM EDTA	86.8 ± 0.1	83.9 ± 0.8	78.4 ± 1.0	71.4 ± 0.7
Rat pool	16.4 ± 0.6	12.1 ± 0.8	13.1 ± 0.6	19.6 ± 1.4
Rat pool + 2 mM CaCl <sub>2</sub>	1.9 ± 0.1	1.3 ± 0.1	2.5 ± 0.2	5.1 ± 3.0
Rat pool + 2 mM EDTA	21.3 ± 0.2	17.1 ± 0.4	17.9 ± 0.4	25.1 ± 1.6
Human 1	76.1 ± 1.9	71.8 ± 1.3	63.4 ± 1.3	56.9 ± 3.2
Human 2	79.1 ± 0.8	71.5 ± 0.4	65.3 ± 3.0	60.6 ± 0.3
Human 3	75.5 ± 6.6	71.9 ± 7.0	67.4 ± 1.2	61.4 ± 0.8
Human pool	78.5 ± 0.5	72.3 ± 0.4	66.8 ± 1.3	55.9 ± 1.5
Human pool + 2 mM CaCl <sub>2</sub>	63.8 ± 0.6	64.4 ± 1.4	56.7 ± 0.4	55.8 ± 0.6
Human pool + 4 mM CaCl <sub>2</sub>	50.0 ± 2.0	45.0 ± 0.7	47.5 ± 0.5	49.5 ± 1.4
Human pool + 2 mM EDTA	84.5 ± 0.5	78.8 ± 0.2	76.8 ± 1.1	73.8 ± 1.8
Human pool + 4 mM EDTA	79.2 ± 0.8	77.9 ± 0.6	78.7 ± 2.9	97.0 ± 5.1

**Table 6-8 Mean unbound fractions and SDs of [<sup>14</sup>C]ibandronate for dog, rat and human**

Matrix	Nominal plasma concentration			
	2000 ng/mL	200 ng/mL	20 ng/mL	2 ng/mL
Dog pool	75.1 ± 1.5	71.6 ± 0.6	64.7 ± 1.3	59.3 ± 3.9
Dog pool + 2 mM CaCl <sub>2</sub>	72.2 ± 1.8	64.8 ± 1.5	58.5 ± 0.7	47.4 ± 4.6
Dog pool + 2 mM EDTA	89.3 ± 1.0	86.1 ± 0.7	83.3 ± 1.2	85.4 ± 1.2
Rat pool	48.5 ± 1.7	32.4 ± 1.3	24.2 ± 2.6	28.9 ± 1.4
Rat pool + 2 mM CaCl <sub>2</sub>	2.6 ± 0.1	2.4 ± 0.1	2.6 ± 0.2	3.1 ± 1.1
Rat pool + 2 mM EDTA	67.0 ± 0.2	63.2 ± 0.8	57.5 ± 3.1	41.9 ± 3.0
Human 1	78.8 ± 2.0	75.7 ± 0.8	73.0 ± 1.1	62.6 ± 2.5
Human 2	79.5 ± 1.2	77.6 ± 0.2	75.0 ± 1.0	59.8 ± 1.2
Human 3	77.5 ± 0.8	75.6 ± 1.3	73.5 ± 0.9	84.2 ± 4.8
Human pool	81.6 ± 0.5	78.0 ± 1.5	73.0 ± 0.7	65.7 ± 2.6
Human pool + 2 mM CaCl <sub>2</sub>	76.0 ± 0.6	72.5 ± 0.6	67.0 ± 0.3	61.8 ± 5.6
Human pool + 4 mM CaCl <sub>2</sub>	69.4 ± 0.9	62.0 ± 3.0	56.2 ± 3.0	52.2 ± 1.5
Human pool + 2 mM EDTA	88.8 ± 1.9	85.4 ± 1.7	81.3 ± 0.9	69.7 ± 0.7
Human pool + 4 mM EDTA	91.7 ± 1.0	93.5 ± 0.7	90.4 ± 1.2	78.6 ± 1.2





**Conclusion:** Zoledronic acid exhibited significant binding to plasma proteins in all tested species. Binding was affected by Ca<sup>2+</sup>, with increased binding at high concentrations of Ca<sup>2+</sup>, and reduced binding at low concentrations of Ca<sup>2+</sup> (presence of EDTA). All species showed a dose-